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Molecular Basis of Paralytic Neurotoxin Action on Voltage-Sensitive Sodium Channels

Annual Summary Report

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In year 1 of this project, progress was made on several objectives:

A. The sites and mechanisms of action on the sodium channel were examined and further defined for three new classes of neurotoxins: Goniopora toxins, Brevetoxins, and Conotoxins.

B. Monoclonal antibodies with high affinity for the mammalian neuronal sodium channel were developed and methods to screen them for activity at neurotoxin binding sites were established, and

C. Site-directed antibodies against defined regions of the amino acid sequence of the sodium channel were prepared and shown to bind at discrete negatively charged subsites on the extracellular surface of the channel that may form part of neurotoxin receptor sites.

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Foreward

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Research Report

In this first year of the project, we have analyzed the effects of several new neurotoxins on voltage-sensitive sodium channels in mammalian neurons, developed the first monoclonal antibodies against the sodium channels of mammalian neurons, and probed the functional effects of polyclonal antisera directed at selected sites in the sodium channel structure.

A. Neurotoxin Action on Sodium Channels

1. Site and Mechanism of Action of Goniopora Toxin (GPT) Goniopora toxin (GPT)1 is a polypeptide of 9,700 daltons isolated from the coral Goniopora spp. (1). The toxin enhances neurally mediated contraction of blood vessels and taenia coli of the guinea pig (2) and has a positive inotropic action on the myocardium (3). The myocardial effects of GPT are due to a prolongation of the action potential caused by a sustained inward current which is reversed by low sodium medium and by tetrodotoxin, a specific inhibitor of voltage-sensitive sodium channels (4). These results suggest that GPT may alter sodium channel gating as part of its mechanism of action. Voltage clamp analysis of the effects of GPT on sodium currents in crayfish giant axons shows that inactivation of sodium channels is markedly slowed (5). In this work, we determined the actions of GPT on sodium currents in neuroblastoma cells using the gigaohm seal voltage clamp technique in the whole cell configuration (6,7) and we examined the interaction of this toxin with previously defined neurotoxin receptor sites on the sodium channel using radioligand binding and ion flux methods (8-11).

Figure 1A shows a family of sodium currents in an N18 neuroblastoma cell before applying GPT. The sodium channels are activated within 1 msec and are inactivated before the end of the 8.75 msec test pulse period. This recording was obtained 50 min after making a seal between the cell membrane and the micropipet. At this time, the exchange of ions between the cell membrane and the micropipet was complete and the amplitude of the sodium currents and the sodium equilibrium potential were constant. Then 50 ml of 1×10^{-5} M GPT were added to the bathing medium approximately 5 mm from the cell. Figure 1B shows a family of sodium currents from the same cell 3 min after adding GPT to the dish. The decay of the sodium currents was markedly slowed so that the currents were no longer inactivated fully at the end of the test pulse period. The maximum sodium currents elicited by all test pulse potentials examined were increased after the addition of the toxin. The increases in current amplitude are shown more clearly in Figure 1C and D
in which the sodium currents elicited by the same test pulse before and after the addition of the toxin are superimposed. The peak currents increased 2.7- and 1.3-fold at test pulse potentials of -20 mV and 0 mV, respectively. The mean increase in 3 cells tested was 2.31 ± 0.34 (S.D.) at -20 mV and 1.39 ± 0.15-fold at 0 mV. Reversal potentials of the currents calculated from the above measurements were unaffected by GPT (+72.3 mV vs +71.5 mV).

The voltage dependence of steady state sodium channel inactivation during 100 msec prepulses is illustrated in Figure 2. In the absence of GPT (O), half maximal inactivation is observed at 62.7 mV. GPT shifts the steady state inactivation curve by 6.0 ± 2.5 mV (S.D., n=3) in the positive direction with little decrease in the steepness of the curve (●). In addition, GPT causes inactivation to be incomplete. Even after 100 msec at -20 mV, more than 5% of the sodium current remains (Figure 2, ●).

The voltage dependence of activation of sodium channels is also altered by GPT. The toxin increases sodium current more at small depolarizations than at large depolarizations (Figure 1C,D). Therefore, when normalized conductance-voltage curves for activation are plotted, GPT shifts the potential for half maximal activation from -10.7 mV to -19.9 mV (Figure 2). The mean value of the negative shift in 3 cells examined was -9.8 ± 2.1 mV (S.D.) A similar effect is observed with other toxins and treatments that slow or block inactivation of sodium channels in N18 cells (18).

Polypeptide neurotoxins from the scorpions Leiurus quinquestriatus or Buthus eupeus the sea anemones Homostichanthus duerdemi or Anemonia sulcata slow the inactivation process of sodium channels and bind to sodium channels in a voltage-dependent manner (7,8,12-16). The effect of GPT on the sodium conductance at different holding potentials was quantitated as the fraction of the sodium conductance remaining 3 msec after the peak (7). The sodium conductance at this time is practically zero in the absence of the toxin (see Figure 1) and is increased in proportion to the number of channels modified by the toxin. The effect of 30 nM or 60 nM GPT becomes progressively smaller when the holding potential is depolarized between -80 and +40 mV. At potentials more negative than -80 mV, the effect of GPT is approximately constant. Figure 3 illustrates apparent K_D values of the toxin calculated from the following relation derived by assuming one-to-one binding of the toxin to sodium channels.

$$K_D = [GPT](F_G/F_G - 1)$$

Here F_G and F_G are the fractions of sodium conductance remaining 3 msec after the peak, and the maximum fraction at saturating GPT, respectively. F_G is 0.75 ± 0.05 (S.D., n=3) following an experiment in the presence of 500 nM GPT (see Figure 1). A linear relationship was obtained between the holding potential and logarithm of the K_D from -80 to +40 mV. Between these potentials the apparent K_D increases e-fold for each 48.3 mV of depolarization.

We examined the effect of ionic composition of the bathing medium on GPT action since preliminary biochemical experiments on GPT action did not detect toxin effects in medium which contained 120 mM choline and only 10 mM Na^+. In these experiments, the recording micropipet contained 140 mM NaF and 20 mM CsCl to allow measurement of outward currents in sodium-free external medium. The holding potential was maintained at -80 mV and, following a prepulse to -120 mV for 100 msec, sodium currents were stimulated by test pulses at membrane potentials from -40 mV to +60 mV in intervals of 10 mV. The presence of GPT did not slow the inactivation process of sodium channels in sodium-free external medium. The time constants of the decay of the sodium current during a test pulse to +10 mV in choline medium and in normal medium were 0.55 ± 0.02 msec (S.D., n=2) and 0.64 ± 0.08 msec (S.D., n=2), respectively. For comparison, outward sodium currents were recorded in choline medium containing 200 nM Lqtx. The inactivation of the sodium channels was markedly slowed in the presence of 500 nM GPT. The slowing of the decay of sodium current by 200 nM Lqtx in the choline medium was similar to that in normal medium. The mean decay time constants were 6.94 ± 0.98 (S.D., n=2) and 7.02 ± 0.86 (S.D., n=2) msec for a test pulse to +10 mV in normal and choline medium, respectively. The modified sodium currents with Leiurus toxin were completely blocked after an addition of TTX. Thus, the effect of GPT on inactivation is lost in Na^+-free medium whereas the effect of Lqtx is retained.

The dependence of the effect of GPT on external sodium ion concentration was determined as follows. Prior to recordings, cells were incubated in choline medium containing 200 nM GPT at 37°C for 30 min as described under Experimental Procedures.

Soon after making a seal between the cell membrane and a micropipet, the amplitude of the outward sodium currents increased as Na^+ from the pipet diffused into the cell. After 20 min, the current amplitude became constant. The concentration of Na^+ in the bathing medium was increased stepwise every 6 min to a final level of 75 mM by adding normal recording medium containing 200 nM GPT. Finally, the Na^+ concentration was raised above 75 mM by addition of 2.5 M NaCl to the bath 2 cm from the cell. Figure 4 illustrates the relationship between Na^+ concentration in the medium and the fraction of sodium conductance remaining 3 msec after the peak of sodium current during a test pulse to +40 mV, as an indicator of the toxin effect on sodium channel inactivation. A linear relationship is observed between 0 and 197 mM Na^+. Thus, Na^+ acts at one or more low affinity binding sites which are not saturated within the physiological range of Na^+ concentration for vertebrates, but may saturate at the higher concentration in sea water, the normal environment for coral toxin action.

Li^+, K^+, Rb^+, and Cs^+ were tested to determine whether they would substitute for Na^+ in sustaining the action of GPT on sodium channels. For Na^+ and for Li^+, which are transported well by the sodium channel, the internal pipet medium was 90 mM CsF, 60 mM CsCl and 10 mM NaCl, and inward currents were measured. For K^+, Rb^+, and Cs^+, which are only slightly permeant, the internal pipet medium was 140 mM NaF, 20 mM CsCl and outward currents were measured. In the absence of GPT, the kinetics of the outward and inward currents flowing through the sodium channels and blocked by TTX in the presence of these cations were similar to those in normal medium or choline medium. The effectiveness of these cations on the action of GPT on sodium channels was compared by estimating the sodium channel conductance remaining at 3 msec after the peak conductance during the test pulse to +10 mV. The middle column in Table 1 shows the fraction of conductance remaining in the presence of 200 nM GPT and the values are normalized to those in normal medium in the right column. Sodium ion has the strongest effect on sustaining the actions of GPT on sodium channels and the order of the effectiveness of the other five cations tested was $Na^+ > K^+ > Rb^+ > Li^+ > Cs^+ >>$ choline$^+$.

The effect of GPT to slow sodium channel inactivation without markedly altering channel activation resembles the actions of a-scorpion toxins and sea anemone toxins which act at neurotoxin receptor site 3 on the sodium channel. In order to determine whether GPT acts at this same receptor site, we examined its effect on specific binding of ^{125}I-labeled scorpion toxin from Leiurus quinquestriatus to sodium channels in N18 cells. Specific binding of ^{125}I-Lqtx was competitively blocked by unlabeled Lqtx with half maximal effect at 1 to 2 nM (Figure 5A) as expected from previous work (8,10). In choline medium, no effects of GPT on specific binding of ^{125}I-Lqtx were observed at concentrations ranging from 10 nM to 1 mM (Figure 5A). In sodium medium, a small inhibition of binding was observed at concentrations above 100 nM. However, this may have resulted from depolarization of the cells since it was reduced by 1 mM tetrodotoxin (data not shown). At the resting membrane potential of N18 cells (-41 mV), the apparent K_D for GPT action on sodium channels is approximately 36 nM (Figure 3). Therefore, the failure of GPT to competitively block binding of ^{125}I-Lqtx at much higher concentrations indicates that its physiological effects on sodium channel inactivation are not mediated by binding at neurotoxin receptor site 3.

We also examined the effect of GPT on ^{125}I-Lqtx binding in synaptosomes (Figure 5B). In this experimental preparation, GPT caused more marked inhibition of ^{125}I-Lqtx binding. Inhibition in Na^+ medium (O) was greater than in choline medium (O). As for N18 cells, the inhibition in Na^+ medium was partially reversed by TTX, suggesting that it may result in part from depolarization. Ten-fold higher concentrations of GPT were required to block ^{125}I-Lqtx binding than to slow inactivation of sodium

channels. Thus, these results with synaptosomes also support the conclusion that GPT action to slow sodium channel inactivation does not require interaction at neurotoxin receptor site 3.

Sea anemone toxins and a-scorpion toxins enhance the persistent activation of sodium channels caused by veratridine, batrachotoxin, and other neurotoxins acting at receptor site 2 on the sodium channel through an allosteric mechanism (9). Since GPT slows sodium channel inactivation like a-scorpion and sea anemone toxins, we have examined whether it would also enhance veratridine-stimulated $^{22}Na^+$ influx into N18 cells. GPT does increase the initial rate of $^{22}Na^+$ influx in the presence of a saturating concentration of veratridine (200 mM). However, it is much less effective than Lqtx causing only a 1.9-fold enhancement at 1 mM. In agreement with the neurotoxin binding results, addition of 1 mM GPT to samples treated with 200 nM Lqtx does not reduce the rate of $^{22}Na^+$ influx appreciably, indicating that it does not compete with Lqtx for binding at neurotoxin receptor site 3.

Two classes of polypeptide neurotoxins which slow sodium channel inactivation have been described previously: the a-scorpion toxins and the sea anemone toxins (reviewed in 17). Although these two families of toxins do not share detectable amino acid sequence homology, they both bind in a voltage-dependent manner to neurotoxin receptor site 3 on the sodium channel and slow channel inactivation. The amino acid sequence of GPT has no homology with the sequence of scorpion or sea anemone toxins. Although GPT also slows sodium channel inactivation, several features of its action distinguish it from the scorpion and sea anemone toxins that have been studied. The failure of GPT to block specific scorpion toxin binding to neurotoxin receptor site 3 in either choline recording medium or normal recording medium at physiologically effective concentrations provides direct support for this conclusion. These results therefore define a new locus on the extracellular aspect of the sodium channel that can selectively alter the process of inactivation. It will be of interest to examine the interactions between GPT and neurotoxins known to act at other receptor sites on the solium channel and to identify the site of GPT action within the structure of the sodium channel protein.

2. Site of Action of Brevetoxin. The toxins from the marine dinoflaggelate Ptychodiscus brevis are candidates for action at a new toxin receptor site. Partially purified preparations of Ptychodiscus brevis toxins cause repetitive firing in the squid giant axon (20), increase the frequency of action potentials in the crayfish nerve cord (21), cause acetylcholine release from nerve endings (22), and depolarize skeletal muscle (23). These effects are blocked by low sodium solutions or by tetrodotoxin (20,22,23), a specific inhibitor of sodium channels in nerve and muscle (24), suggesting that P. brevis toxins may affect sodium channel function.

Two nonprotein toxins from P. brevis have been purified to homogeneity by high pressure liquid chromatography and designated T46 and T47 on the basis of chromatographic properties (25). More recently, these toxins have also been purified by repeated flash chromatography and renamed brevetoxin A and brevetoxin B, respectively (26). Brevetoxin B has been determined by X-ray crystallography (26) to be a rigid ladderlike structure consisting of 11 contiguous fused ether rings with a molecular weight of 894. In our previous work (27), we have examined the effects of brevetoxin A, the most potent of the pure toxins (25,26), on sodium channels in cultured neuroblastoma cells. We found that this toxin enhances the persistent activation of sodium channels by neurotoxins acting at neurotoxin receptor site 2 such as veratridine and batrachotoxin. The value of $K_{0.5}$ for activation by both toxins was reduced and the maximum fraction of sodium channels activated by the partial agonist veratridine was markedly increased.

In view of the similarity of brevetoxin A action to the actions of the a-scorpion and sea anemone toxins, we propose that it also acts as a heterotropic allosteric modulator of sodium channel activation which reduces the value of the allosteric constant, M_{RT}, governing channel activation (8). By reducing the energy required to activate a channel, brevetoxin A would increase the probability of channel activation. This mechanism might lead to the repetitive action potentials observed in nerve preparations incubated with brevetoxins. Further experiments will be required to determine if this action is sufficient to account fully for the effects of the toxins.

Because the enhancement of alkaloid toxin activation by brevetoxin resembles the effects of the polypeptide toxins, it was of interest to determine whether brevetoxin binds at neurotoxin receptor site 3 on the sodium channel. Specific binding of ^{125}I-labelled scorpion toxin to sodium channels in neuroblastoma cells was measured in the presence of increasing brevetoxin A concentrations from 10 to 1000 ng/mL. Brevetoxin A had no effect on the binding of scorpion toxin demonstrating that it does not occupy neurotoxin receptor site 3 on the sodium channel (27).

We have also tested binding of brevetoxin A to neurotoxin receptor site 1 on the sodium channel. Specific binding of [^{3}H]saxitoxin to sodium channels in neuroblastoma cells was measured at concentrations of brevetoxin A from 10 to 1200 ng/mL. Brevetoxin A did not block saxitoxin binding and may have slightly enhanced it (27). Thus, brevetoxin A does not bind to neurotoxin receptor sites 1 or 3 in exerting its effects on sodium channels.

Sodium channels in synaptosomes have the same properties as those in neuroblastoma cells with respect to neurotoxin binding and action (28,29). Moreover, since the number of sodium channels per mg of protein is much greater in synaptosomes, they have proven to be a more useful preparation for many biochemical studies of channel properties. Binding of neurotoxins to receptor site 2 on the sodium channel can be studied using [^{3}H]batrachotoxinin A 20-a-benzoate ([^{3}H]BTX-b) as a site-specfic probe (19,21). Specific binding of [^{3}H]BTX-b is quite low when tested alone but is increased 20-fold when measured in the presence of scorpion toxin (19) as expected from previous ion flux studies demonstrating allosteric interaction between neurotoxin receptor sites 2 and 3 (8). Under these conditions, specific binding to sodium channels accounts for 75% of total [^{3}H]BTX-b binding, has a K_D of 70 nM, and is completely blocked by appropriate concentrations of unlabeled batrachotoxin, veratridine, and aconitine (19).

Our ion flux experiments (27) predict that brevetoxin A, like a-scorpion toxins, should enhance specific [^{3}H]BTX-b binding. To examine this possibility, we measured [^{3}H]BTX-b binding to sodium channels in synaptosomes after incubation for 30 min at 36° in the presence of 10 nM [^{3}H]BTX-b plus increasing concentrations of brevetoxin A from 10 to 1000 ng/mL. Nonspecific binding was measured in the presence of 300 mM veratridine and subtracted from the results. The data in Figure 6 show that brevetoxin A does indeed increase specific [^{3}H]BTX-b binding. A 4.3-fold increase is observed at 1000 ng/mL brevetoxin A with a half-maximal effect at 35 ng/mL. These results correspond closely to the data from our ion flux experiments with neuroblastoma cells. At 100 ng/ml, brevetoxin A increased [^{3}H]BTX-b binding 3-fold. Half-maximal effects of brevetoxin A are observed at 35 ng/mL for [^{3}H]BTX-b binding and 50 ng/mL for $^{22}Na^+$ influx (27). This close correlation supports the hypothesis that brevetoxin A acts by an allosteric mechanism to enhance alkaloid toxin binding and action at neurotoxin receptor site 2.

Our results show that brevetoxin A effects on sodium channels are half-maximal in the range of 35 to 50 nM. Since the toxin is active at a low concentration, it is likely that it acts at a specific receptor site on the sodium channel protein rather than by a more indirect mechanism such as alteration of general membrane properties. Previous work established the existence of neurotoxin receptor sites 1 through 4 on the sodium channel (31). The work presented previously (27) and our new results suggest that the brevetoxins do not bind at neurotoxin receptor sites 1 and 3 on the sodium channel. Additional experiments in which brevetoxins were shown to enhance the binding of ^{125}I-labeled Centruroides suffusus suffusus toxin II to neurotoxin receptor site 4 on the sodium channel eliminate this as the site of brevetoxin action. We conclude that the brevetoxins act at a new neurotoxin receptor site on the sodium channel which we propose to designate site 5. Since brevetoxin causes repetitive firing and modulates sodium channel activation by veratridine, it is likely that this receptor site is located on a region of the sodium channel involved in voltage-dependent gating. The brevetoxins provide the first potential probes of this new receptor site on the sodium channel.

3. Actions of Conotoxins on Sodium Channels. We have carried out a preliminary characterization of the electrophysiological effects and sites of action of two new neurotoxins from the piscivorous marine snails Conus striatus (32) and Conus geographus (33) in collaboration with Dr. Y. Ohizumi. Striatoxin, a glycopeptide of unknown structure, slows sodium channel inactivation. Its action is voltage-dependent. In these respects it resembles neurotoxins acting at receptor site 3 on the sodium channel. Geographotoxin II is a 22 residue polypeptide containing hydroxyproline and multiple disulfide bridges. It blocks sodium channels in muscle specifically with no effect on sodium channels in nerve at comparable concentrations. In cultured muscle cells, it blocks the tetrodotoxin-sensitive sodium channels characteristic of adult muscle but not the tetrodotoxin-insensitive sodium channels characteristic of denervated and uninnervated muscle. Thus, this toxin has a remarkable selectivity among different sodium channel classes.

Like Goniopora toxin described above, the snail toxin striatoxin also blocks sodium channel inactivation without altering toxin binding at neurotoxin receptor sites 1 and 3. Thus, it too defines a new region of the sodium channel which is important for the process of inactivation. It will be considerable interest to determine whether these two classes of neurotoxins share a common site of action.

Geographotoxin blocks sodium channels in a manner similar to saxitoxin and tetrodotoxin. We find that it competes with these toxins for binding at neurotoxin receptor site 1 on the sodium channel of skeletal muscle, but not of nerve. Thus, features of neurotoxin receptor site 1 must differ betwen nerve and muscle. This toxin will be valuable to probe tissue specific structural elements of sodium channels.

B. Production of Monoclonal Antibodies Against the Sodium Channel.

The sodium channel is a highly conserved protein which has proven to be a poor antigen in the hands of most investigators. Thus, both polyclonal and monoclonal antibodies prepared against sodium channels from eel electroplax crossreact poorly with sodium channels in nerve and muscle (34,35). Occasional monoclonal antibodies which do crossreact generally have low affinity (36, but see 37 for an exception). Polyclonal and monoclonal antibodies against sodium channels from rat muscle crossreact poorly with nerve (38,39). Polyclonal antisera against rat brain sodium channels do not crossreact with rat muscle and actually cross react with sodium channels in peripheral nerve relatively poorly (40). Antibodies that alter neurotoxin action on sodium channels have been observed in only one case (37). These various studies from several

laboratories all lead to the conclusion that antibodies are usually produced against unique tissue- and species-specific epitopes on the sodium channels rather than conserved regions present in all sodium channels. Since the functional elements of sodium channels and the toxin receptor sites which modulate channel function are highly conserved, isolation of antibodies against these regions will require screening of large numbers of independent antibody isolates or use of new experimental approaches.

In the first year of work on this proposal, we have established methods for production and screening of monoclonal antibodies using both *in vivo* and *in vitro* immunization techniques and have isolated the first high affinity monoclonal antibodies directed against sodium channels in mammalian neurons. For *in vivo* immunization, mice were immunized with repeated does of highly purified sodium channels until a high titer of anti-sodium channel antibodies was produced. Splenic lymphocytes were then dissociated from excised tissue and fused with clone SP2-derived myeloma cells using polyethylene glycol. Replicating hybrid cells were selected in HAT medium and screened for secretion of anti-sodium channel antibodies in a single step using a specific radioimmune assay for sodium channels (41). This single-step screening procedure with highly purified sodium channel reagents was found to be quite important in accurately identifying antibody secreting clones of interest within a few days after fusion and rapidly cloning them. Using this approach, we have isolated several hybridoma cell lines which secrete a high titer of monoclonal anti-sodium channel antibodies. Representative clones have been injected into mice for production of larger amounts of antibody in ascites fluids. Immunoprecipitation of sodium channels by ascites fluids from two of these clones is illustrated in Figure 7. Half-maximal immunoprecipitation of sodium channels in our radioimmune assays is produced by .01 to .03 mL of these ascites fluids.

We are currently in the process of analyzing the effects of these monoclonal antibodies on neurotoxin binding and action on sodium channels. Our initial experiments with binding of saxitoxin and Leiurus scorpion toxin have not identified major effects. We plan to continue this program of production and functional screening of monoclonal antibodies.

The immune response to conserved regions of proteins is often suppressed by poorly understood mechanisms in vivo. It has been suggested that these suppression phenomena can be avoided if antigen is presented to lymphocytes *in vitro* in cell cultures (42). Thus, *in vitro* immunization may provide an approach to increasing the probability of isolation of antibodies against conserved functional regions of sodium channels. We have now established the *in vitro* immunization method in our laboratory. In two trials of immunization of freshly dissociated cultured lymphocytes by this method (42), we have identified multiple hybridoma clones producing antibodies against the sodium channel, but none of these have proven to have high affinity for the channel. These clones have not been characterized further. We intend to apply this method more extensively in the next year using assay procedures designed to detect high affinity antibodies directed against neurotoxin receptor sites soon after fusion to form hybridomas.

C. Site-directed Antibodies that Alter Neurotoxin Action

We have now determined the complete amino acid sequence of the a subunit of the sodium channel from rat brain by cDNA sequencing methods (43). Most of the functional sites of the sodium channel reside on this subunit. We have selected regions of the a subunit that are likely to be components of receptor sites for positively charged sodium channel neurotoxins like saxitoxin and scorpion toxin by identifying regions of concentrated negative charge located on the extracellular surface of the channel. The corresponding polypeptides have been synthesized, covalently coupled to carrier

proteins, and injected into rabbits to produce polyclonal antisera. One of these antisera has now been characterized in detail. The binding of these antibodies to the sodium channel is illustrated in Figure 8A. The high affinity antibodies in the serum bind the purified sodium channel in the nM concentration range. A Scatchard analysis of the antibody binding data suggests a K_D value of 4.8 nM for the high affinity antibodies (Figure 8B). Thus, these antibodies have high enough affinity to be valuable probes of the structure of the sodium channel.

In order to determine whether these antibodies bind to native sodium channels in intact membranes and to establish whether their binding site is on the intracellular or extracellular face of the membrane, the antiserum was incubated with intact or lysed synaptosomes, bound antibodies were removed by sedimentation, and the remaining unbound antibodies in the supernatant were quantitated by radioimmune assay. The results of Figure 9 show that both intact and lysed synaptosomes bind these site-directed antibodies equivalently. Thus, the antibodies bind to an epitope that is equally available in intact and lysed synaptosomes. This epitope is therefore present on the extracellular surface of the native sodium channel. This represents the first negatively charged site identified on the extracellular surface of the channel and it is therefore a prime candidate for a neurotoxin receptor site. These antibodies do not affect [^{3}H] saxitoxin binding to sodium channels indicating that this epitope does not form part of neurotoxin receptor site 1. Further studies to test their effects on binding and action of other neurotoxins on sodium channels are in progress.

Antisera against other defined channel regions are under development. When these become available, they will be analyzed to determine the location of their epitope and their effects on neurotoxin binding in order to define a functional map of the sodium channel.

TABLE 1

Comparison of the effects of different ions on GPT action.

The ratio of the sodium conductance remaining at 3 msec after the peak to the peak conductance was measured in experiements like the one described in the text for 2 to 4 cells for each cation. Mean ratios and standard deviation are given along with values normalized to those in Na^+ medium.

Cation	$\frac{G_{Na}\ (t = 3\ msec)}{G_{Na}\ (peak)}$	Normalized Values
Na+	0.60 +0.07	1.0
K+	0.51 + 0.17	0.84
Rb+	0.40 + 0.02	0.67
Li+	0.35 + 0.04	0.58
Cs+	0.16 + 0.02	0.26
Choline	0.02 + 0.02	0.04

FIGURE 1

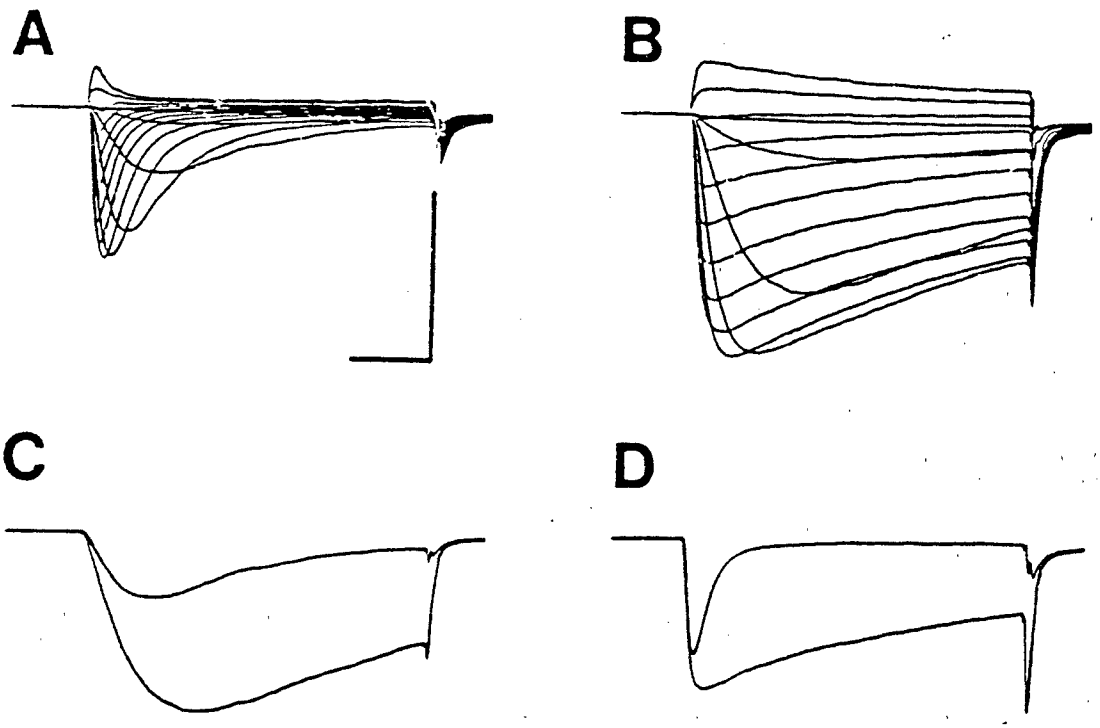


FIGURE 2

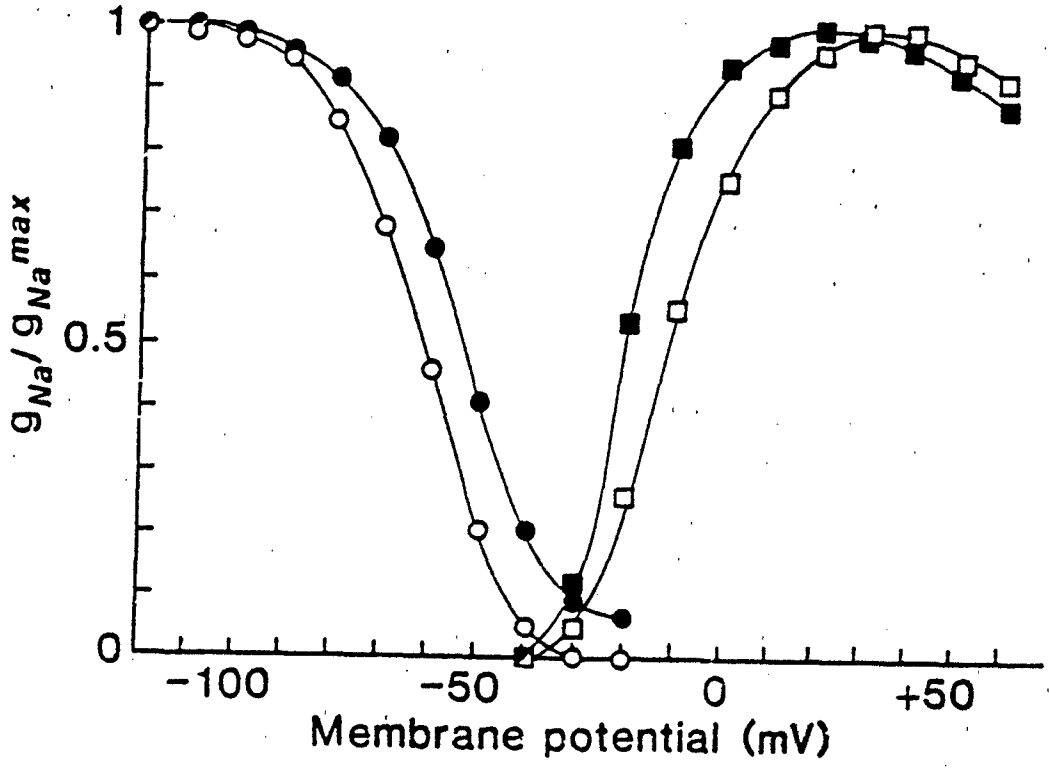


FIGURE 3

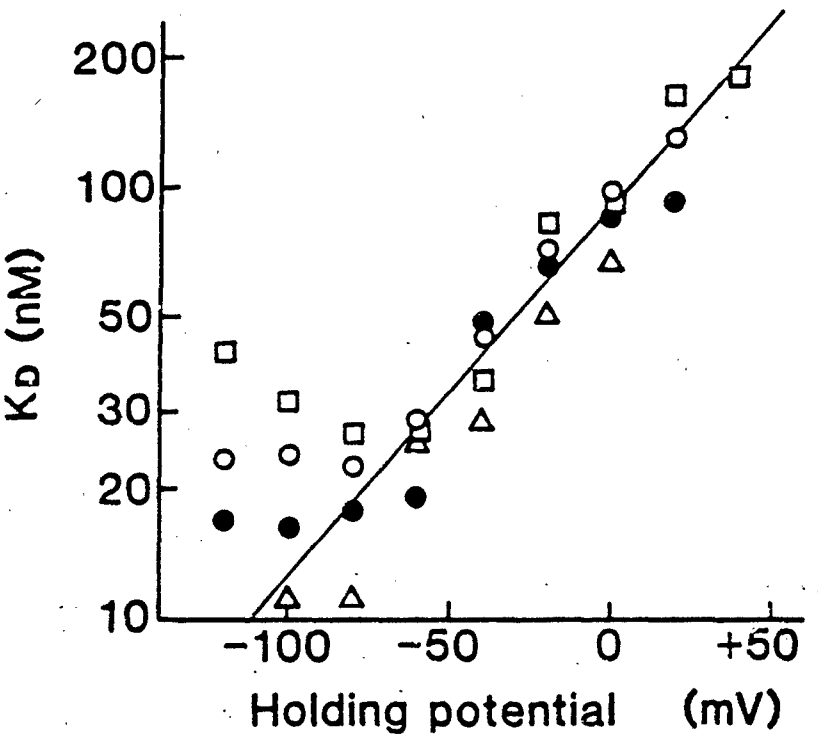


FIGURE 4

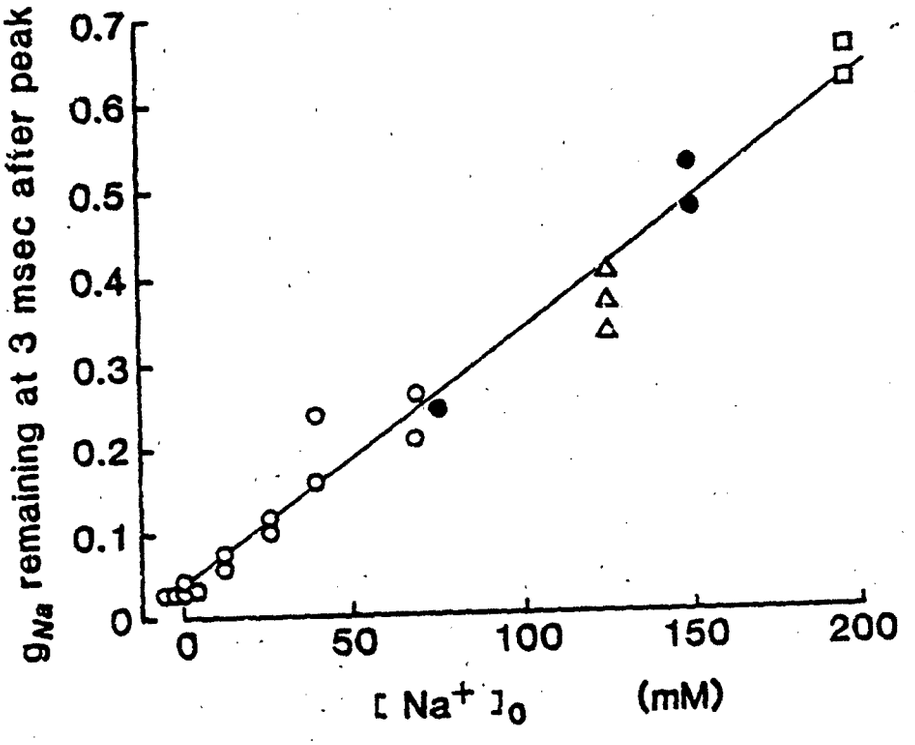

g_{Na} remaining at 3 msec after peak
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
200
[Na^+]$_o$ (mM)

FIGURE 5

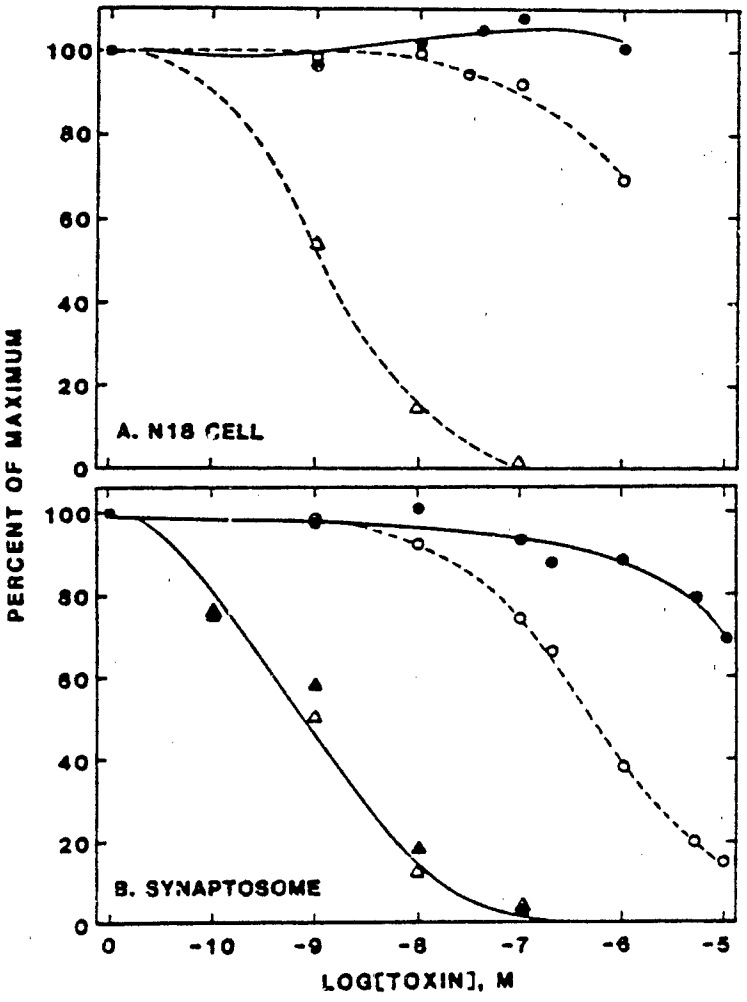

PERCENT OF MAXIMUM
100
80
60
40
20
0
A. N18 CELL
B. SYNAPTOSOME
0
-10
-9
-8
-7
-6
-5
LOG[TOXIN], M

FIGURE 6

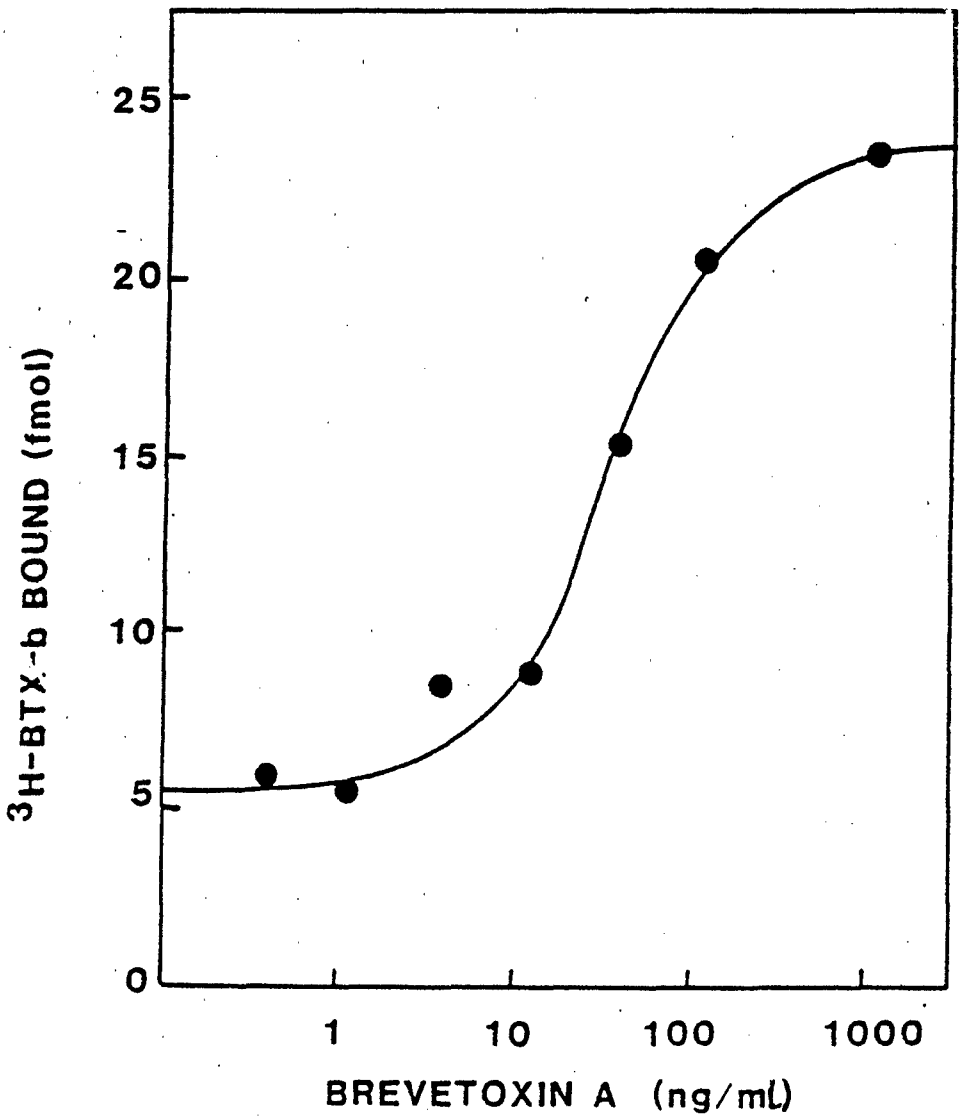

25
20
15
10
5
0
^{3}H-BTX-b BOUND (fmol)
1
10
100
1000
BREVETOXIN A (ng/mL)

FIGURE 7

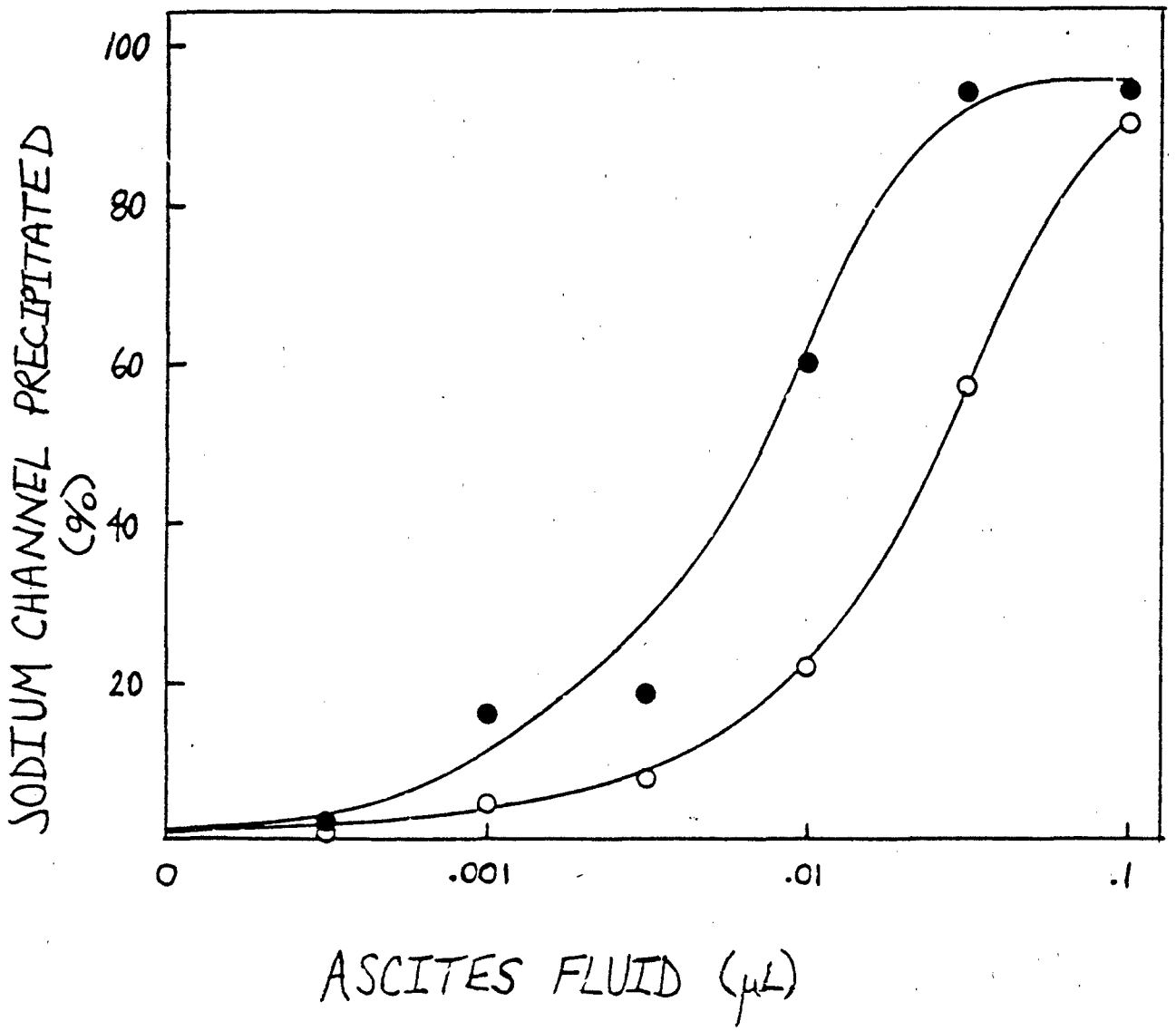

SODIUM CHANNEL PRECIPITATED (%)
100
80
60
40
20
0
.001
.01
.1
ASCITES FLUID (μL)

FIGURE 8

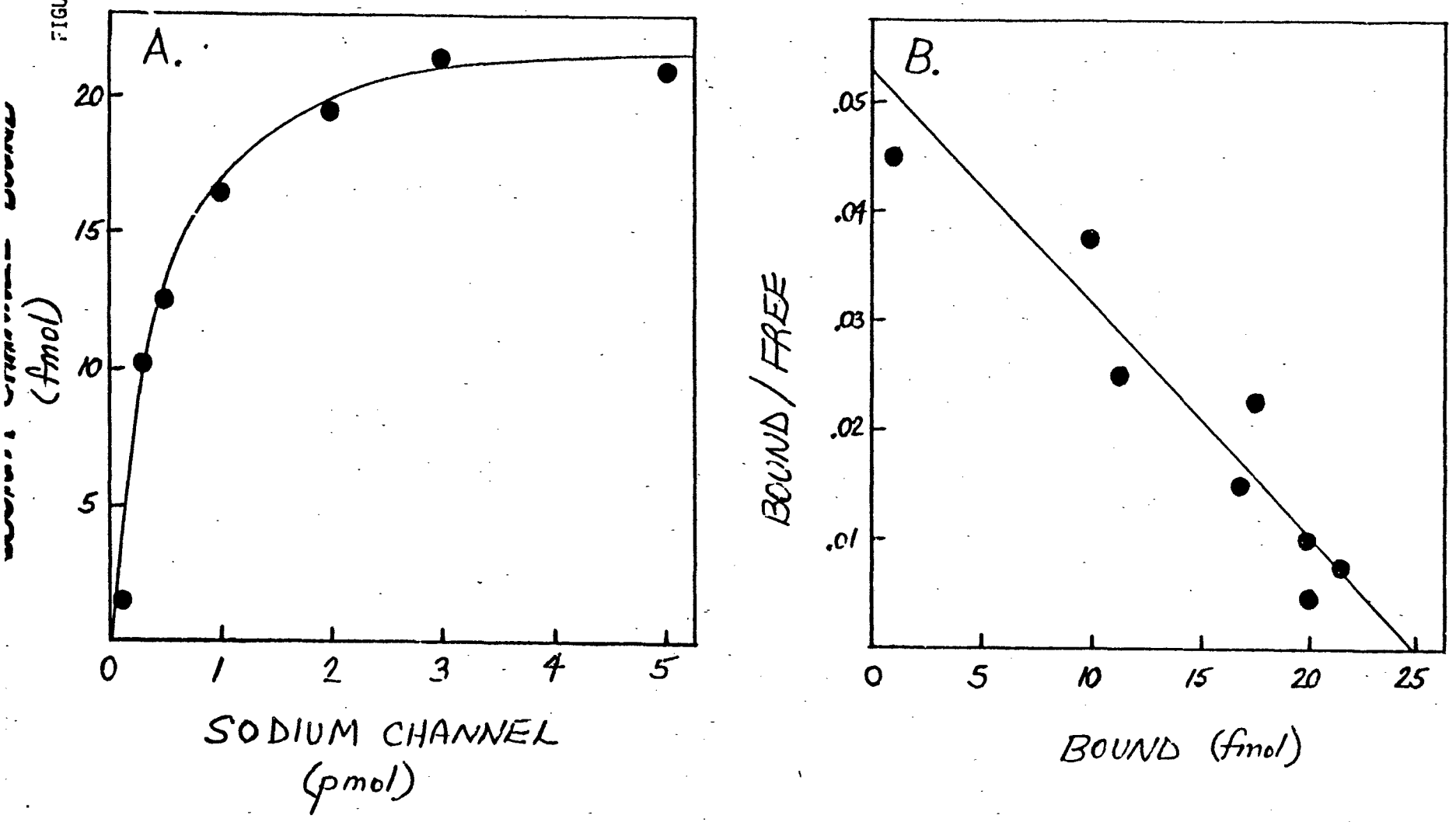
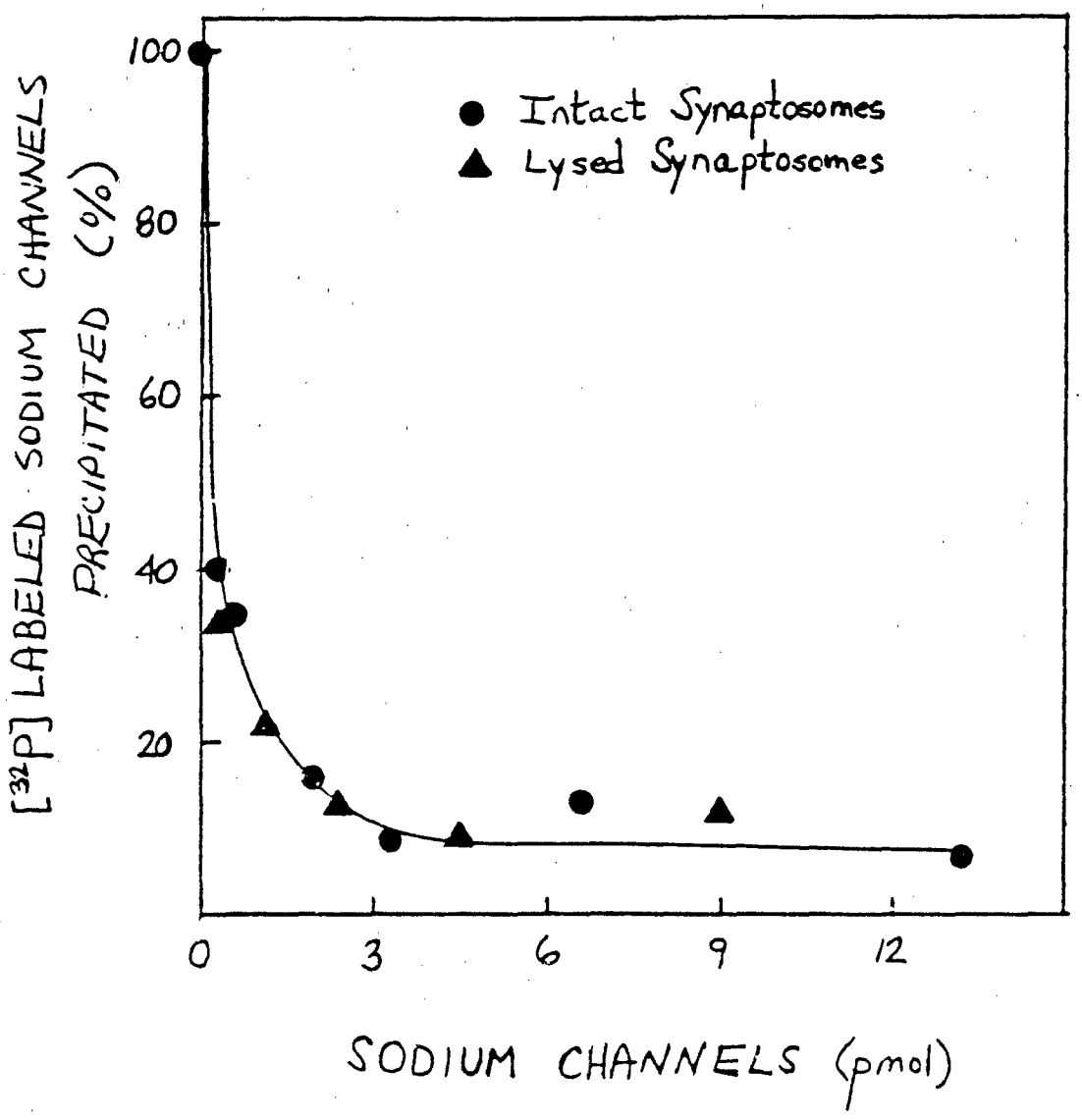


FIGURE 8

FIGURE 9


[32P] LABELED SODIUM CHANNELS PRECIPITATED (%)
100
80
60
40
20
0
3
6
9
12
SODIUM CHANNELS (pmol)
Intact Synaptosomes
Lysed Synaptosomes

Literature Cited

1. Hashimoto, Y., and K. Ashida. Screening of toxic corals and isolation of a toxic polypeptide from Goniopora spp. Publication of Seto Marine Biological Laboratory 20:703-711 (1973).

2. Muramatsu, I., M. Fumiwara, S. Ikushima, and K. Ashida. Effect of Goniopora toxin on guinea-pig blood vessels. Naunyn-Schmiedeberg's Arch. Pharmacol. 312:193-197 (1980).

3. Fujiwara, M., I. Muramatsu, H. Hidaka, S. Ikushima, and K. Ashida. Effect of Goniopora toxin, a polypeptide isolated from coral, on electromechanical properties of rabbit myocardium. J. Pharmacol. Exp. Ther. 210:153-157 (1979).

4. Noda, M., I. Muramatsu, and M. Fujiwara. Effect on Goniopora toxin on the membrane currents of bullfrog atrial muscle. Naunyn-Schmiedeberg's Arch. Pharmacol. 327:75-80 (1984).

5. Muramatsu, I., M. Fujiwara, and T. Narahashi. Effects of Goniopora toxin, a polypeptide isolated from coral, on crayfish giant axon. Proceedings of the Eighth International Congress of Pharmacology, Tokyo. p. 318.

6. Hamill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. Pflugers Arch. 391:85-100 (1981).

7. Gonoi, T., B. Hille, and W.A. Catterall. Voltage clamp analysis of sodium channels in normal and scorpion toxin-resistant neuroblastoma cells. J. Neurosci. 4:2836-2842 (1984).

8. Catterall, W. A. Membrane potential-dependent binding of scorpion toxin to the action potential Na^+ ionophore. Studies with a toxin derivative prepared by lactoperoxidase-catalyzed iodination. J. Biol. Chem. 252:8660-8668 (1977).

9. Catterall, W.A. Activation of the action potential Na^+ ionophore by neurotoxins. An allosteric model. J. Biol. Chem. 252:8669-8676 (1977).

10. Ray, R., C.S. Morrow, and W.A. Catterall. Binding of scorpion toxin to receptor sites associated with voltage-sensitive sodium channels in synaptic nerve ending particles. J. Biol. Chem. 253:7307-7313 (1978).

11. Tamkun, M.M., and W.A. Catterall. Ion flux studies of voltage-sensitive sodium channels in synaptic nerve ending particles. Mol. Pharmacol. 19:78-86 (1981).

12. Catterall, W.A. Binding of scorpion toxin to receptor sites associated with sodium channels in frog muscle. correlation of voltage-dependent binding with activation. J. Gen. Physiol. 74:375-391 (1979).

13. Mozhayeva, G.N., A.P. Naumov, E.D. Nosyreva, and E.V. Grishin. Potential-dependent interaction of toxin from venom of the scorpion Buthus eupeus with sodium channels in myelinated fibre. Biochim. Biophys. Acta 597:587-602 (1980).

14. Lawrence, J.C. and W.A. Catterall. Tetrodotoxin-insensitive sodium channels. Binding of polypeptide neurotoxins in primary cultures of rat muscle cells. J. Biol. Chem. 256:6223-6229 (1981).

15 Kryshtal, O.A., Yu. V. Osipchuk, G.B. Elyakov, and E.P. Kozlovskaya. Action of neurotoxin from the sea anemone Homostichanthus duerdemi on inward sodium current in mammalian neurons. Neurophysiology 14:303-309 (1982)

16. Warashina, A., and S. Fujita. Effect of sea anemone toxins on the sodium inactivation process in crayfish axons. J. Gen. Physiol. 81:305-323 (1983).

17. Catterall, W.A. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. Ann. Rev. Pharmacol. Toxicol. 20:15-43 (1980).

18. Gonoi, T., K. Ashida, W.A. Catterall, and B. Hille. Na channel activation shifts after inactivation removal in neuroblastoma cells. Biophys. J. 47:33a (1985).

19. Catterall, W.A., C.S. Morrow, G.B. Brown, and J.W. Daly. Binding of batrachotoxinin A 20-a-benzoate to a receptor site associated with sodium channels in synaptic nerve ending particles. J. Biol. Chem. 256:8922-8927 (1981)

20. Westerfield, M., J. W. Moore, Y.S. Kim, and G.M. Padilla. How Gymnodinium breve red tide toxins (s) produces repetitive firing in squid axons. Am. J. Physiol. 232:C23-29 (1977).

21. Parmentier, J.L., T. Narahashi, W.A. Wilson, N.M. Trieff, V.M.S. Ramanujam, M. Risk and S.M. Ray. Electrophysiological and Biochemical characteristics of Gymnodinium Breve toxins. Toxicon 16:235-244 (1978).

22. Gallagher, J.P., P. Shinnick-Gallagher. Effect of Gymnodinium breve toxin in the rat phrenic nerve diaphragm preparation. Br. J. Pharmac. 69:367-372 (1980).

23. Shinnick-Gallagher, P., Possible mechanisms of action of Gymnoidinium breve toxin at the mammalian neuromuscular junction. Br. J. Pharmac. 69:373-378 (1980).

24. Narahashi, T., Chemicals as tools in the study of excitable membranes. Physiol. Rev. 54:813-889 (1974)

25. Risk, M., Y.-Y. Lin, V.M.S. Ramanujam, L.L. Smith, S.M. Ray and N.M. Trieff. High pressure liquid chromatographic separation of two major toxic compounds from Gymnodinium breve Davis. J. Chromat. Sci. 17:400-405 (1979)

26. Lin, Y.-Y., M. Risk, S.M. Ray, D. Van Engen, J. Clardy, J. Golik, J.C. James, and K. Nakanishi. Isolation and Structure of brevetoxin B from the "Red Tide" dino flagellate Ptychodascus Brevis (Gymnobindinium Breve). J. Am. chem. Soc. 103:6773 (1981).

27. Catterall, W.A., and M. Risk. Toxin T46 from Ptychodiscus brevis (formerly Gymnodinium breve) enhances activation of voltage-sensitive sodium channels by veratridine. Mol. Pharmacol. 19:345-348 (1981).

28. Kreuger, B.K., M.P. Blaustein, and R.W. Ratzlaff. Sodium channels in presynaptic nerve terminals. J. Gen. Physiol. 76:287-313 (1980).

29. Tamkun, M.M., and W.A. Catterall. Ion flux studies of voltage-sensitive sodium channels in synaptic nerve-ending particles. Mol. Pharmacol. 19:78-86 (1981).

30. Brown, G.B., S.C. Tieszen, J.W. Daly, J.E. Warnick, and E.X. Alburquerque. Batrachotoxinin-A 20-a-benzoate: a new radioactive ligand for voltage sensitive sodium channels. Cell. Molec. Neurobiol. 1:19-40 (1981)

31. Catterall, W.A., The molecular basis of neuronal excitability. Science 223:653-661 (1984).

32. Kobayashi, J., H. Nakamura, Y. Hirata, and Y. Ohizumi. Isolation of a cardiotonic glycoprotein, striatoxin, from the venom of the marine snail Conus Striatus. Biochem. Biophys. Res. Comm. 105:1389-1395 (1982)

33. Sato, S., H. Nakamura, Y. Ohizumi, J. Kobayashi and Y. Hirata. The amino acid sequences of homologous hydroxproline-containing myotoxins from the marine snail Conus geographus venom. FEBS Letters. 155:277-280 (1983).

34. Moore, H., L. Fritz, M. Raftery, and J. Brockes. Isolation and characterization of a monoclonal antibody against the saxitoxin-binding component from the electric organ of the eel Electrophorus electricus. Proc. Natl. Acad. Sci. USA. 79:1673-1677 (1982)

35. Fritz, L., and J. P. Brockes. Immunochemical properties and cytochemical localization of the voltage-sensitive sodium channel from the electroplax of the eel (Electrophorus electricus). J. Neurosci. 3:2300-2309 (1983).

36. Meiri, H., I. Zeitoun, H. Grunhagen, V. Lev-Ram, Z. Eshhar, and J. Schlessinger. Monoclonal antibodies associated with sodium channel block nerve impulse and stain nodes of Ranvier. Brain Research. 310:168-173 (1984).

37. Barhanin, J., H. Meiri, G. Romey, D. Pauron, and M. Lazdunski. A monoclonal immunotoxin acting on the Na^+ channel, with properties similar to those of a scorpion toxin. Proc. Natl Acad. Sci. USA. 82:1842-1846 (1985).

38. Haimovich, B., E. Bonilla, J. Casadei, and R. Barchi. Immunocytochemical localization of the mammalian voltage-dependent sodium channel using polyclonal antibodies against the purified protein. J. Neurosci. 4:2259-2268 (1984).

39. Casadei, J. M., R. Gordon, L. Lampson, D. Schotland, and R. Barchi. Monoclonal antibodies against the voltage-sensitive Na^+ channel from mammalian skeletal muscle. Proc. Natl. Acad. Sci. USA. 81:6227-6231 (1984).

40. Wollner, D., and W.A. Catterall. Antigenic differences among the voltage-sensitive sodium channels in the peripheral and central nervous systems and skeletal muscle. Brain Research. 331:145-149 (1985).

41. Costa, M., and W.A. Catterall. Cyclic AMP-dependent phosphorylation of the a submunit of the sodium channel in synaptic nerve ending particles. J. Biol. Chem. 259:8210-8218 (1984).

42. Reading, C., Theory and methods for immunization in culture and monoclonal antibody production. J. of Immun. Methods. 53:261-291 (1982).

43. Auld, V., J. Marshall, A. Goldin, A. Dowsett, W.A. Catterall, N. Davidson and R. Dunn. Cloning and characterization of the gene for alpha subunit of the mammalian voltage-gated sodium channel. J. Gen Physiol.85:10a (1985).